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Quantification of the binding affinity of a specific hydroxyapatite binding peptide

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ABSTRACT

The genesis of bone and teeth involves highly coordinated processes, which involve multiple cell types and proteins that direct the nucleation and crystallization of inorganic hydroxyapatite (HA). Recent studies have shown that peptides mediate the nucleation process, control HA microstructure or even inhibit HA mineralization. Using phage display technology, a short peptide was identified that binds to crystalline HA and to HA-containing domains of human teeth with chemical and morphological specificity. However, the binding affinity and specific amino acids that significantly contribute to this interaction require further investigation. In this study, we employ a microfluidic chip based surface plasmon resonance imaging (SPRi) technique to quantitatively measure peptide affinity by fabricating a novel 4 layer HA SPR sensor. We find the peptide (SVSVGMKPSPRPGGGK) binds with relatively high affinity ($K_D = 14.1 \mu M \pm 3.8 \mu M$) to HA. The independently measured amino acid fragment SVSV seems to impart a significant contribution to this interaction while the MKPSP fragment may provide a conformational dependent component that enhances the peptides affinity but by itself shows little specificity in the current context. These data show that together, the two moieties promote a stronger synergistic binding interaction to HA than the simple combination of the individual components.

1. Introduction

Normal and pathological mineralization of calcium phosphates are critical biological processes impacting both the medical and tissue engineering fields. One prominent polymorph of calcium phosphate essential during the normal mineralization processes of both teeth and bone matrix formation is hydroxyapatite (HA). Similarly, however, pathological derivatives of this mineral, such as carbonate-substituted hydroxyapatite, are implicated in osteoarthritis as well as other forms of degenerative arthritis [1]. As with all biological processes, a sensitive balance exists at multiple levels of regulation in which introduction or absence of one or more mediators can tip the balance in favor of normal or pathological biomineralization. The formation of the hydroxyapatite phase during normal biomineralization processes, such as osteogenesis and odontogenesis, is regulated by a complex orchestration of multiple cell types and numerous proteins. In the context of bone formation (osteogenesis), proteins such as bone sialoprotein, osteonectin, osteocalcin, and collagen I interact with HA to template the localized mineralization of HA, giving rise to the microstructures observed in bone which strongly contribute to its mechanical properties [2–6]. Similarly in tooth formation (odontogenesis), dentin matrix protein and statherin are implicated in regulating mineralization of calcium phosphate precursors into HA [7–9].

Despite the wealth of literature devoted to studying the bone biomineralization process during osteogenesis and identifying potential mediators, a coherent mechanism remains elusive due in part to the limitation of the current measurement methods. Indirect measurement techniques such as the von Kossa assay, ortho-cresolphthalein complexone, and Alizarin red [10–12] methods are destructive and purely endpoint measurements. Further, each method only identifies the presence of calcium and is not sensitive to the chemical composition or to the specific polymorphs of calcium phosphate such as HA [13]. Robust, new probes with both chemical and morphological specificity, perhaps coupled to a fluorescent

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reporter are necessary to advance our understanding of the HA mineralization process.

Small peptides are finding widespread utility as material specific probes. Several synthetic peptides containing amino acid sequences from HA interacting proteins, like those listed above, have been shown to promote or inhibit HA crystallization and interact directly with HA [2,8,9,14–16]. In efforts to improve upon nature, genetically engineered peptides that recognize other inorganic materials including Au, Ag, and Pt have been identified using combinatorial screening methods like phage display [17–22]. Recently, a peptide sequence that bound preferentially to HA over its amorphous calcium phosphate precursor was identified with phage display; however, measuring a quantitative binding affinity proved challenging [23]. Subsequent peptides that bind to calcium phosphate substrates have been found but again quantitative affinity measurements and insight into the mechanism of binding remain incomplete [24].

Investigators address these issues by using quartz crystal microbalance (QCM), atomic force microscopy (AFM) and surface plasmon resonance (SPR) [23,25–27]. SPR and SPR imaging have developed into major quantitative techniques for measuring the adsorption kinetics of small peptides onto a diverse set of material substrates [28,29]. However, a HA chip for use in the SPR imaging configuration was not previously available. In this effort, we used SPRi in conjunction with a novel HA coated sensor to quantify the binding affinity of a previously identified hydroxyapatite-binding peptide [23]. We further examine which amino acid segments contribute to the binding to aid future probe development while also lending insights into the potential mechanism(s) of binding.

2. Materials and methods

2.1. Peptides

Previously, phage display technology was used to identify the peptide sequence (HA-1, SVSVGMKPSPRPGGGK-biotin) which binds with high affinity to crystalline hydroxyapatite, as well as the hydroxyapatite-containing portions of human teeth [23]. This sequence, a scrambled sequence (Scram, PKGPSVMGGR-biotin) and two fragment sequences (HA-2, SVSVGGK-biotin; HA-3, VSMKPSPGGGK-biotin) were synthesized using standard *FMOC* solid phase peptide synthesis techniques with double coupling (Advanced ChemTech Apex 396), purified by dialysis in de-ionized water (molecular mass (MW) cutoff 1000 g/mol, cellulose membrane, Spectra) then dried and stored at −20 °C. Each peptide sequence contained a C-terminal biotin (*-b*) group. The dried peptides were re-hydrated in a 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer made with ultra-pure de-ionized water (Milli-Q, Millipore).

2.2. Fabrication and characterization hydroxyapatite substrate

Hydroxyapatite (HA) substrates consisted of a base, glass substrate (25 mm × 50 mm, Fisher Scientific) which was cleaned by O_2 plasma treatment for 5 min (Plasma Prep Jr., SPI Supplies West Chester PA). To this base, thin films of Cr (0.6 nm), Ag (50 nm), TiO_2 (2.8 nm) and hydroxyapatite (10 nm–20 nm) were patterned by sequential layer sputtering in an argon atmosphere at 25 °C (Denton Vacuum Discovery-550). A custom target was used to create the thin layer of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, Cerac Specialty Inorganics). The Cr and Ag layers were deposited using Direct Current (DC) mode, while TiO_2 and HA were deposited with Radio Frequency (RF) mode. The thicknesses of the individual layers were determined by both material deposition rates and Fresnel estimates. Surface morphology and uniformity were visualized by Atomic Force Microscopy (AFM, Veeco Dimension 3100) and Scanning Electron Microscopy (SEM, Zeiss Ultra-60). To prevent surface charging and enhance contrast during SEM imaging, samples were briefly sputtered with Au. The four films were also deposited on polished Si substrates (Wafer World, Inc) for SEM imaging and X-ray photoelectron spectroscopy (XPS) analysis. XPS was used to conduct an elemental analysis of the hydroxyapatite substrate. Spectra were obtained on a Kratos AXIS Ultra DLD spectrometer with a monochromatic Al x-ray source (1486.7 eV) operating at 140 W under 1.0×10^{-9} Torr vacuum. Measurements were performed in hybrid mode using electrostatic and magnetic lenses, and the take-off angle was 0° (angle between the sample surface normal and the electron optical axis of the spectrometer), which yields a maximum sampling depth of approximately 8 nm [30]. Atomic concentrations were calculated from survey spectra, collected over a binding-energy (BE) range from 1100 to 0 eV using a pass energy of 160 eV, energy resolution of 0.2 eV, and a 500 ms dwell time. High resolution (region) scans were collected using a pass energy of 20 eV and a sweep time of 60 s. The number of sweeps for survey and region scans was 2 and 3, respectively. A flood gun was used for charge neutralization, and all spectra were shifted with respect to the C 1s peak at 284.6 eV. Two spectra were acquired for each sample. A clean Si wafer specimen was also measured as a control. Peak areas for Ca 2s, P 2p, O 1s and C 1s were fitted using a Levenberg–Marquardt algorithm assuming a linear background (CasaXPS software).

2.3. Micro-channel construction

A multi-channel microfluidic device consisting of six, parallel flow channels measuring 40,000 μm × 300 μm × 120 μm (L × W × H) was constructed using standard soft lithographic techniques [31,32]. Briefly, Su8-50 photoresist (Micro Chem) was spun cast onto a Si wafer and then selectively polymerized using ultraviolet light shone through a lithographic mask containing the design, creating positive micro-channel features. Polydimethylsiloxane (PDMS, Dow Corning Sylgard 184) was then cast onto the patterned Si wafer, cured at 70 °C overnight, cut to size (50 mm × 25 mm) and then attached to the HA substrate to create an SPR compatible flow-cell.

2.4. Surface plasmon resonance (SPR) imaging setup

A custom SPR imaging system Kretschmann configuration, SPR BioSystems was built around a sapphire prism ($n = 1.77$) and 514 nm light emitting diode (LED) source (LE-1G, WT&T Inc). The reflected intensity (R) of both p- and s-polarized LED light (R_p and R_s) was collected with a cooled CCD camera (Retiga 2000RV, QImaging). Computer imaging software (StreamPix NorPix Inc.) was used to capture images of 1) reflected intensities from (R_p and R_s) illumination at different angles of incident and 2) images of reflected p-polarized light (R_p) at a fix incident angle as a function of time. ImageJ (Rasband, W.S., National Institute of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) was used to extract from the collected images, the average intensities of a central region of interest (ROI, 600 pixels × 12 pixels) within an individual flow channel. Using capture method 1 and ImageJ, the characteristic reflectivity (R_p/R_s) vs. incident angle (θ) curve of each channel was constructed. This was done first for incident angles of 57°–85° at increments of 0.1°. Based on the position of the resonance angle (θ_R) incident θ at which the reflectivity (R_p/R_s) is a minimum), a second reflectivity (R_p/R_s) curve was constructed from a narrower range of incident angles (typically 66°–78° at 0.05°). The principally parabolic curve was fit to a 6th order polynomial using least square regression and the derivative of this function was calculated to identify the SPR imaging angle (θ_i), the angle that yields maximum sensitivity in R_p/R_s changes and corresponds to the inflection point of the R_p/R_s curve and is $< \theta_R$. For real time measurements of R_p using capture method 2, the incident angle was fixed at θ_i (typically 74°–76°). In this case, reflectivity (R_p/R_s) is calculated based on an initial measurement of R_s (θ_i) when determining the reflectivity curve and assumed to remain constant during the time course over which R_p is collected. This assumption appears to be valid because R_s did not change significantly over the duration of the experiment, presumably due to the stability of the LED illumination source.

2.5. Peptide binding experiments and SPR image analysis

All micro-channels were treated with a 5 mg/mL bovine serum albumin (BSA) solution for ≈ 1 h at 25 °C to minimize the non-specific adsorption of peptides to the PDMS during binding experiments. The PDMS micro-channels were then washed with de-ionized water (18 MΩ cm^{-1}) and dried. Finally, the channels were secured to the HA substrate and optically coupled to the prism of the SPR setup with immersion oil ($n = 1.522$). Peptide solutions were prepared in 25mM HEPES buffer (pH 7.4) at several concentrations (200, 100, 50, 25, and 10 μM). It should be noted that buffers containing sodium chloride (i.e., PBS or TBS buffers) could not be used with the HA substrate due to the ability of such buffers to penetrate the outer layers and react with the silver layer. Syringe pumps (Braintree Scientific Inc.) were used to introduce only HEPES buffer for 10 min–15 min at a flow rate of 2.5 μL/min to establish a baseline. After characteristic reflectivity curves were collected and the imaging angle set, a specific peptide concentration was flown into individual channels at a flow rate of 2.5 μL/min. Once an initial positive change in the average reflected intensity (p-polarized light) was observed, the peptide was infused for ≈ 45 s (≈1 channel volume) and then flow was stopped by turning the pump off and closing a syringe valve, creating a pseudo-static condition for steady-state measurements and allowing the system to approach equilibrium. Images of R_p were collected every 5 s for 20 min–30 min at 400 ms exposure time and converted to reflectivity (R_p/R_s) within the ROI. A baseline reflectivity $[R_p/R_s]_B$ value, time average of R_p/R_s values over the first 5 min, was subtracted from the R_p/R_s data set to yield the change in reflectivity ($\Delta(R_p/R_s)$), which is equivalent to the typical % R reported in the literature divided by 100%. This data treatment typically resulted in very small $\Delta(R_p/R_s)$ values that fluctuated around zero until peptide adsorption began (shortly after 5 min), indicated by increasing positive $\Delta(R_p/R_s)$ values. These positive $\Delta(R_p/R_s)$ values were reported and are theoretically proportional to the amount of peptide binding to the HA surface. From this data, peptide binding kinetics and steady-state behavior in